Multigenerational effects of carbendazim in *Daphnia magna:* from a subcellular to a population level

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Abstract

Anthropogenic activities such as the use of pesticides may affect in some way aquatic biota populations, due to potential agricultural runoffs or disposals. Carbendazim is one example of a widely used fungicide with a high potential to end up in aquatic ecosystems through runoff. Deleterious effects observed at the individual level are possibly explained by changes in homeostasis at cellular and both can then be used to predict effects at the population level. In the present study, an isoclonal population of *Daphnia magna* (clone k6) was exposed to concentration that mimics relevant levels of carbendazim in the environment during twelve generations. The effects of carbendazim on biochemical biomarkers (cholinesterase, catalase and glutathione *S*-transferase), lipid peroxidation and energy-related parameters (carbohydrates, lipids and proteins jointly with energy available and energy consumption), parental longevity, and population growth (*r*) were assessed in some generations. The long-term exposure to carbendazim presented no effect on the intrinsic rate of natural increase (*r*) of adult *D. magna.* However, daphnids longevity decreased at the F12 when compared to daphnids from control. Cholinesterases, glutathione *S*-transferase and lipid peroxidation showed differences between the exposed and non-exposed populations. However, for catalase and energy related-parameters no differences were observed between these two populations. Natural variability was observed throughout the test period, under control conditions, within the twelve generations. Overall, carbendazim induced some effects at the subcellular level that were translated to longevity, but latter vanishing in terms of population effects.

Key words *Daphnia magna*, multigenerations, carbendazim, biochemical biomarkers, energy reserves, DNA damage

1. Introduction

Pesticides are extensively used in nowadays agriculture practices across the world (Ecobichon, 2001), leading to a potential continuous or pulses release to aquatic systems by runoff and consequently to long-term exposures. It is expected that organisms may be exposed throughout several generations, thus assessing multigenerational effects is of utmost relevance. Although some multigenerational studies have been already carried out with pesticides (Brausch and Smith, 2009; Liess et al., 2013), no clear conclusions have been drawn regarding long-term effects at the population level.

Although environmental relevant concentrations of chemical compounds are generally low with no associated acute toxicity observed, such conditions can still cause sublethal effects in time, reducing organisms' fitness. This might be related, for instance, with the accumulation of damage at a sub-organismal level, such as DNA damage, changes in enzymatic pathways and unbalanced internal energy budget, which may later affect trait related endpoints (*e.g.* growth or reproduction) (De Coen and Janssen (2003b)).

Understanding effects at a subcellular level is an important tool in toxicology to discuss effects at the individual level. Biomarkers may be considered measures of initial changes in response to toxic compounds and can provide more information regarding changes in sensitivity upon a long-term exposure of a population. A biomarker approach thus can help to better depict modes of action of chemical compounds, which later relates to effects at higher levels of organization.

Considering the above mentioned, the general aim of the present study was to assess the sublethal effects of a long-term exposure to a pesticide in *D. magna*, by using a multigenerational approach. For that, carbendazim was chosen as a model fungicide tested over twelve generations of *D. magna,* and the effects occurring at subcellular, individual and population related levels were assessed and discussed to infer to any linkage between responses at different levels of biological organization. The possibility to work with clonal lineages and generate genetically identical offspring, due to parthenogenetic reproduction, makes the water flea *Daphnia magna* a good species to test effects at the multigenerational level (Hebert and Ward, 1972). Additionally, population studies can be simulated at the laboratorial scale in order to predict effects at this higher organizational level.

Carbendazim (CBZ: methyl-2-benzimidazole carbamate) has been used for many years as a fungicide in several agricultural crops, including potatoes, strawberries, onions, wheat, oranges, among others (EU Pesticide Database, 2016) and consequently it is likely to be released from spring to autumn. CBZ is the active breakdown product of benomyl, which is also a systemic fungicide (Davidse, 1973). Emissions to aquatic systems include spray-drift or run-off from crops and soils (after rainfall events), that may occur in a cadence of continuity or pulses (WHO, 1993). CBZ was considered persistent in the water layer (Cuppen et al., 2000), and the maximum reported concentrations was 4.5 µg/L in surface waters of the basin of the Traiguén river in Chile (Palma et al., 2004). In addition, in a previous study, a multigenerational test showed that DNA damage (genotoxicity) increased throughout generations of *D. magna* exposed to CBZ (Silva et al., 2017). Therefore selecting several biomarkers that may provide additional information on other mechanisms induced by CBZ under long term exposures may be useful to understand effects at the individual and population level. Several biomarkers were selected: cholinesterase (ChE) activity, a well-known target site of carbamate pesticides, which inhibits its activity triggering neurotoxic effects in *D. magna* (Barata et al., 2004); catalase (CAT) as an antioxidant enzyme (Brown et al., 2004); glutathione *S*-transferases (GST) which is related with biotransformation and antioxidant defense (Hyne and Maher, 2003); lipid peroxidation (LPO) rate, which is associated with cell damage (Barata et al., 2005); and energy reserves related parameters.

2. Materials and methods

2.1 Test organism and test chemicals

The water flea *D. magna* Straus clone K6 (originally from Antwerp, Belgium) was obtained from continuous culture maintained in a laboratory at the University of Aveiro (Portugal) and cultured in American Society for Testing and Materials moderated-hard-water medium (ASTM, 1980) (temperature 20±1^{0}C; photoperiod 16h:8h (light:dark)). Daphnids were fed with the microalga *Raphidocelis subcapitata* at a concentration of 3x10^5 cells/mL and supplemented with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.).

A stock solution of carbendazim (CAS No. 10605-21-7, 99.4% purity, Bayer Crop Science) was prepared in ASTM medium and used to maintain the multigenerational test. Chemical analyses were performed to confirm concentrations of CBZ in the test

medium at Marchwood Scientific Services, Southampton, UK. CBZ was analysed by Liquid Chromatography-Mass Spectrometry (LCMS-MS) using the QuERCHERS (quick, easy, cheap, effective, rugged, safe) method (details for the chemical analyses can be found in supplementary material).

2.2 Multigenerational experimental setup

For the multigenerational approach the concentration of 5 µg/L of CBZ was chosen based on the results from a reproduction test, where an equivalent no-observed-effect-concentration (NOEC) was derived (Silva et al., 2015). The experimental design of the multigenerational experiment is shown in Figure 1 and will be further described in the following subtopics (where F represents generation). For additional details please check Figure 1 SD.

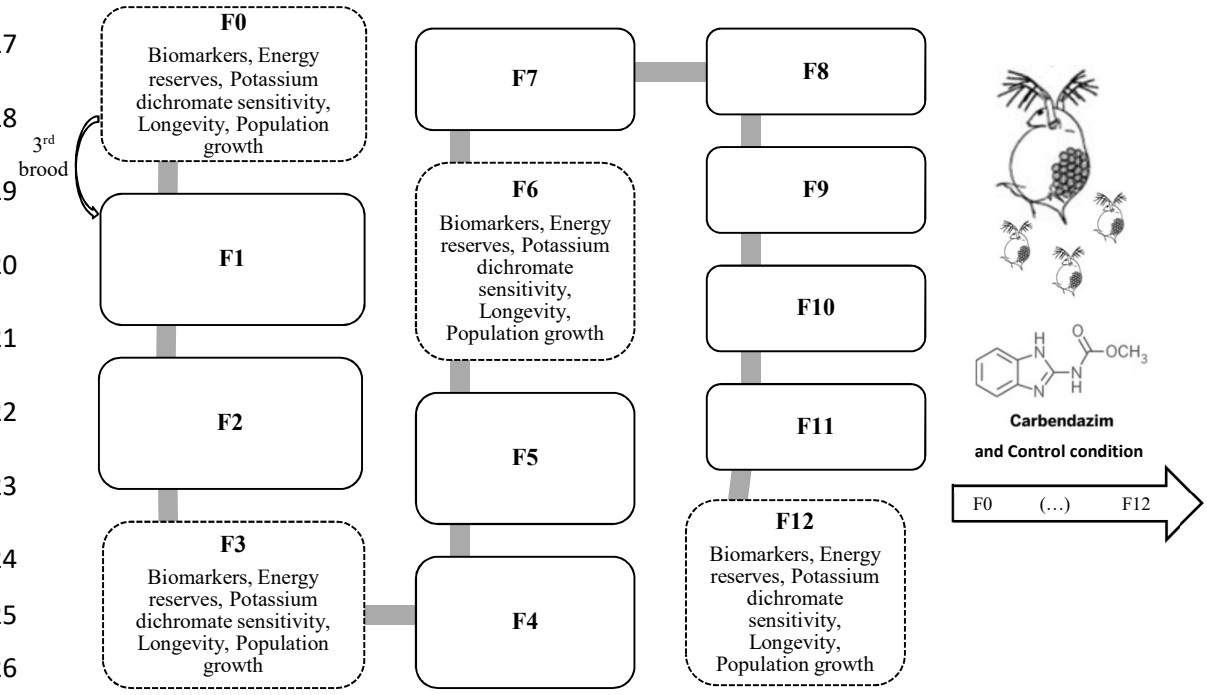

Figure 1. Multigenerational experimental approach with *Daphnia magna*. Each box represents a generation and the respective endpoints evaluated or bioassays carried out. F represents generation.

During the multigenerational experiment, an isoclonal population of *D. magna* was exposed to 5 µg/L of CBZ throughout 12 generations, with the intention to look forward and test the effects in a large temporal time scale. The choice for the 12 generations allows the comparison to previous results available (Silva et al., 2017) along with the fact that this fungicide may be present during several months in the field,

as its application can occur for different agricultural crops and at different times during the year (EU Pesticide Database, 2016). Simultaneously, a second isoclonal population of daphnids was maintained under clean medium and used as control. The population of daphnids that was maintained in a control/clean condition (ASTM, *R. subcapitata* and organic extract but no CBZ) will be designated throughout the study as Dph_Clean and population of daphnids exposed to CBZ (ASTM, *R. subcapitata,* organic extract and CBZ) as Dph_CBZ.

D. magna multigenerational bioassays were carried out in triplicate using glass vessels (1L volume capacity) with 20 daphnids each (<24h neonates), for both isoclonal populations. Each replicate consisted in ASTM medium with *R. subcapitata* (concentration of $3x10^5$ cells/mL) and organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.), spiked with CBZ in the case of the Dph_CBZ population. The medium was completely renewed three times a week. When neonates were not required for any parameter evaluation, they were discarded and removed in a daily basis. Each subsequent generation was always initiated by using third brood neonates (<24h) of the previous one and maintained in the same conditions (either Dph_Clean or Dph_CBZ).

In the F0, F3, F6 and F12 generations several parameters were evaluated (see sections below). Adults' reproduction was reported in time, along with the time for the first brood, and also survival and longevity. Their broods were used to evaluate offspring fitness through sensitivity tests and biochemical markers. In order to control differences in daphnids' responses from sensitivity variations/biological variation in organisms (Loureiro et al., 2010; Novais and Amorim, 2015) all endpoints were simultaneously assessed in neonates from Dph_Clean and Dph_CBZ throughout generations.

2.2.1. Individual and Population level endpoints

The total number of neonates till the fifth brood (21 days) and parental survival were recorded for both populations and the intrinsic rate of natural increase (*r*) calculated, using the Euler Lotka equation (Lokta, 1913):

(Equation 1) $\sum_{x=0}^{x} lx\ mx\ e^{-rx} = 1$

where *lx* is the proportion of individuals surviving to age *x*, *mx* is per-capita fecundity, and *x* represents days.

Parental longevity (lifespan in days) was also recorded. Adult daphnids were kept under the same conditions as in the test, until they die. The day of death was recorded and time-response relationship, using the 50% lethal time (LT_{50}) values, was determined.

Additionally, a sensitivity test with potassium dichromate ($K_2Cr_2O_7$) was performed with multigenerational offspring, according to the OECD procedure (OECD, 2004). In brief, neonates (<24h old) were exposed to a concentration range of $K_2Cr_2O_7$ for 24h and their immobilization recorded.

2.2.2. Subcelullar level endpoints

All biochemical analyses were carried out in 7 days old organisms. For that, and from each treatment, *D. magna* neonates (<24h) were randomly sampled from the three multigenerational replicates, in order to accomplish a 7 day exposure design with five replicates, of 16 organisms each, for the enzymatic activities and LPO measurements, and three replicates with 20 organisms each, for the energy reserves assays (Figure 1 SD). These exposures were maintained in similar conditions as their parental exposures.

For the enzymatic determinations and LPO, organisms were collected to 1.5 mL *Eppendorfs,* with maximum media removal, shock frozen in liquid nitrogen and then stored at -80ºC until analyses. Prior to analysis, samples were prepared using an adapted protocol described by Ferreira *et al.* (2010). ChE activity was measured according to the Ellman method (Ellman et al., 1961) adapted to a 96 well microplate as described in Guilhermino et al. (1996). CAT activity was determined based on the methodology described by Claiborne (1985) adapted to microplate (Ferreira et al., 2015). GST activity was determined according to the method described by Habig et al. (1974) adapted to microplate (Frasco and Guilhermino, 2002). Details for all enzyme analyses are presented in supplementary data. LPO was determined as described by Ohkawa et al. (1979) and Bird and Draper (1984), adapted to microplate, by measuring the production of thiobarbituric acid-reactive substances (TBARS) at 535 nm. For details please check the supplementary data.

Energy reserves were measured using a protocol adapted from Ferreira et al. (2015) previously described by De Coen and Janssen (1997). Total proteins, carbohydrates and lipid contents; energy consumption (Ec), as electron transport

activity – ETS and available energy (Ea) were determined (protocol details can be found as well in supplementary data), and calculated as:

(Equation 2) Ec = ETS activity (mJ/org/min)

(Equation 3) Ea = carbohydrates + lipids + proteins (mJ/org)

2.3 Statistical Analysis

The mean value of the intrinsic rate of natural increase (*r*) was determined using the Jackknife method (Pestana et al., 2010; Taberner et al., 1993). The 50% lethal time (LT_{50}) values were calculated using a nonlinear regression with a three-parameter logistic function using SigmaPlot v11.0 software (Systat Software Inc., 2008). To compare the LT_{50} values obtained for Dph_Clean and Dph_CBZ, a generalized likelihood ratio test was applied using statistical package SPSS (SPSS 20.0.0, 2011). Normality was assessed using the Shapiro-Wilk test and homoscedasticity using Levene's equal variance test (Systat Software Inc., 2008). GST and Ec data were square-root transformed to correct for normality.

Significant differences between exposure (Dph_Clean and Dph_CBZ) and generations (time) were checked for all endpoints (except longevity) using a two-way ANOVA with Bonferroni post-test; and generations (time) and exposure were used as fixed factors. The Two-way ANOVA were performed in SigmaPlot v11.0 software as well (Systat Software Inc., 2008). The R-squared (R^2) was calculated by dividing the sum of squares of each factor and of their interaction by the total sums of squares of the two-way ANOVAs (Hullett and Levine, 2003), to evaluate the percentage of variance accounted for each factor in the ANOVAs.

3. Results and Discussion

3.1.Chemical analysis

The results of the chemical analysis showed that in the ASTM, CBZ concentration decreased in time, with a decay rate (*K0*) of 0.03/hour (st. error= 0.005), showing that only 18% of the initial concentration (7.2 µg/L) left after 48h (described already in Silva et al. (2015)).

3.2. Individual and Population level endpoints

3.2.1. Intrinsic rate of natural increase (r)

The intrinsic rate of natural increase (*r*) is a representative endpoint that can provide information at the population level by traducing offspring production and adults' survival, within a time frame, into an indication of population growth (Buhl et al., 1993). This endpoint (*r*) usually represents a more sensitive parameter than considering only the number of neonates, because it integrates the reproduction output, number of mothers, number of broods and time (days) to the brood release. This enables also to bridge the gap on extrapolations from individuals to populations. However, this pattern was not observed in the present study, with no significant differences for *r* between Dph_Clean and Dph_CBZ (two-way ANOVA, $F_{1,23} = 2.78$, $p>0.05$) and neither an interaction between exposure and generations (two-way ANOVA, $F_{3,23} = 3.12$, $p>0.05$) (Fig. 2 and Table 1 SD). Similar findings by Zalizniak and Nugegoda (2006) showed no clear effects of chlorpyrifos on three successive generations of *Daphnia carinata* for the intrinsic rate of natural increase (*r*). This might be related with a compensation between survival, fecundity and maturation time (Zalizniak and Nugegoda, 2006), and potential an differential allocation of energy.

Some variability on data from non-exposed daphnids (Dph_Clean) was also observed (Fig. 2), which might be related with non-fully controlled exposure conditions such as food quality and/or small variations in room temperature. Although all conditions are intended to be constant a slight inherent variability is likely to occur. In the work of Clubbs and Brooks (2007), a slight variance in the mean number of neonates/intrinsic rate of population growth was also shown even in controls of F0 and F1. In the present study, the difference obtained in *r* from F0 to F3 may be related to the time for the first brood release. While F0 daphnids released their first brood at day 8 (average), the F3 daphnids only released their first brood at day 10 (average). Although it is acceptable that daphnids release their first brood between 8 and 10 days old, this may provide some slight differences when evaluating datasets. Howsoever, as previously described, endpoints were compared between Dph_Clean and Dph_CBZ within generations, to control differences in organisms sensitivity as well (Loureiro et al., 2010).

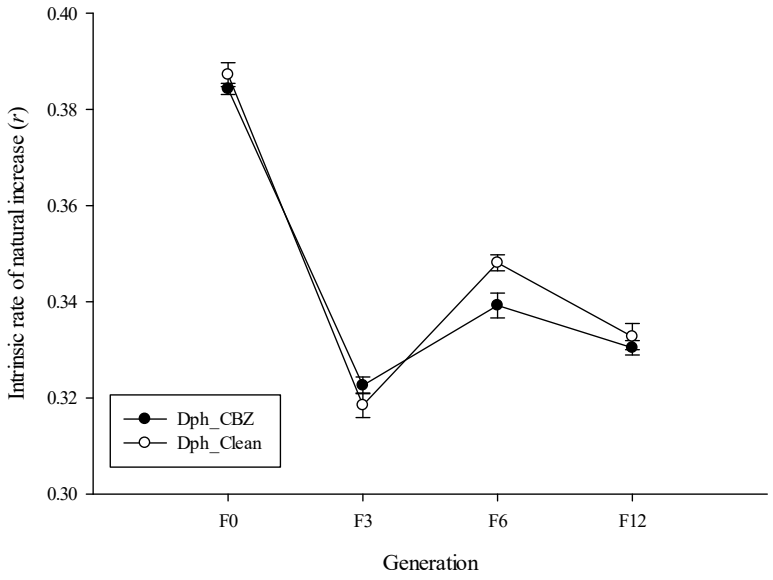

Figure 2. Intrinsic rate of natural increase (*r*) of *Daphnia magna* populations exposed throughout 12 generations to control conditions (Dph_Clean, white dots) and to carbendazim (Dph_CBZ, black dots) Data are expressed as mean values and standard error (n=3).

3.2.2. Longevity in parental organisms

In the F0 generation, the pattern for longevity was similar between Dph_Clean and Dph_CBZ (Fig. 3), which was reinforced by the similar LT_{50} values: 61.97 days and 60.52 days, respectively (Table 2 SD), with no significant differences in slopes of the probit regressions between both LT_{50} values ($X^2_{df=1}$ = 0.91, p>0.05). After twelve generations (F12), longevity was affected by CBZ, with a LT_{50} value of 57.87 days, significantly lower than the 76.18 days for Dph_Clean ($X^2_{df=1}$= 676.2, p<0.05) (Table 2 SD). Throughout the generations the LT_{50} values were always in the same order in the Dph_CBZ population, however in the F12 the longevity in Dph_CBZ was lower when compared to F12 Dph_Clean and this should be taken into account. Survival has been evaluated in daphnids in multigenerational tests, however this assessment is usually carried out only until the 21 d (corresponding to a standard reproduction test with *Daphnia*) (Chen et al., 2013; Sánchez et al., 2004; Tanaka and Nakanishi, 2002). Chen et al. (2013) observed that the pesticide pentachlorophenol caused an earlier mortality in F2 comparing with F0 daphnids, representing an enhanced toxic effect in the F2 generation. Increase in sensitivity due to a continuous exposure probably results from chemical bioaccumulation or transgenerational reductions in fitness (Kimberly and Salice, 2014). In addition, in the present study the *r* did not show any significant

differences between and within treatments and generations, probably representing an higher energy investment by daphnids on reproduction upon exposure to CBZ in detriment of an investment in long-term survival.

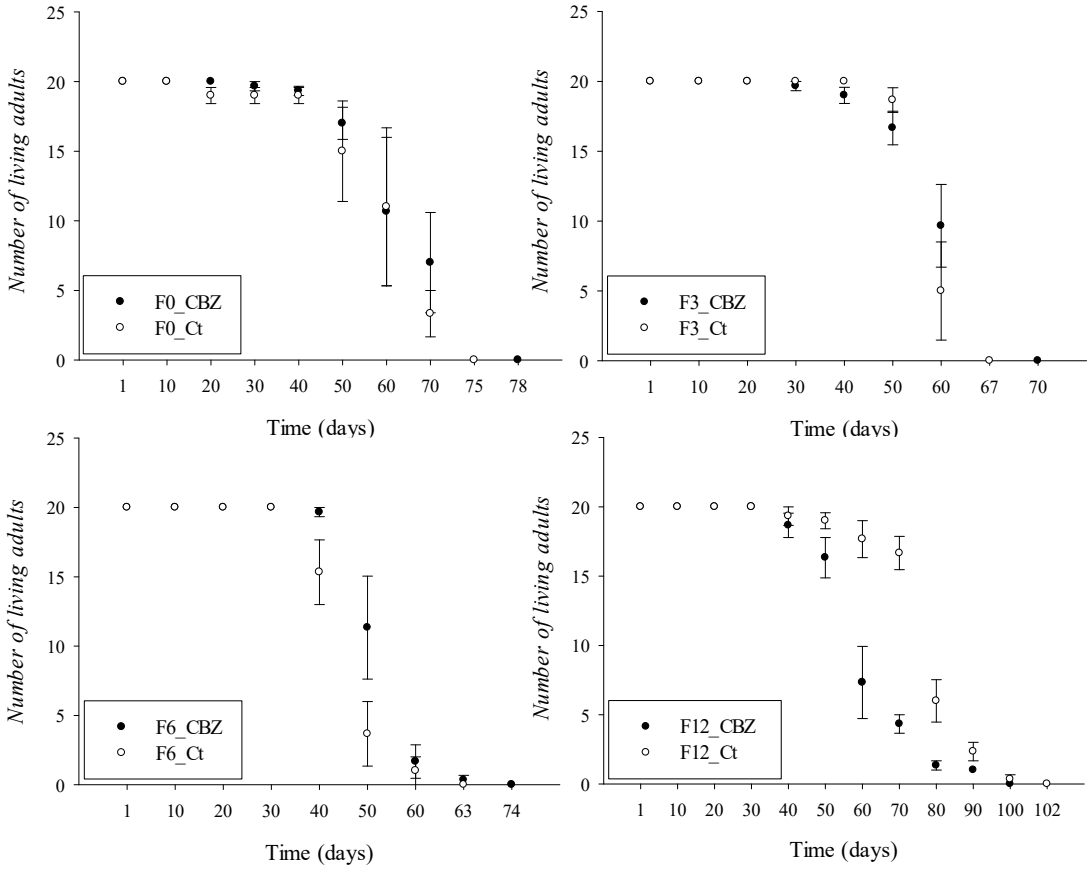

Figure 3. Longevity of *Daphnia magna* populations in control conditions (Dph_Clean, white dots) and exposed to carbendazim (Dph_CBZ, black dots) for several generations: F0 generation, F3 generation, F6 generation and F12 generation (n=3). Longevity (in days) is expressed as mean values of live adults and standard error, for every 10 days.

3.2.3. Neonates fitness- sensitivity test

Throughout the multigenerational experiment, the two isoclonal populations (Dph_Clean and Dph_CBZ) were relatively synchronised in their reproduction (with a difference of only some hours).The physiological conditions of *D. magna* were measured by looking at their sensitivity towards exposure to the reference chemical potassium dichromate. The 24h-EC50 values obtained in control daphnids (Dph_Clean) and in carbendazim (Dph_CBZ) from F0, F3, F6 and F12 were always within the

307 recommended range of 0.6 mg/l to 2.1 mg/l (Table 3 SD) (EN ISO 6341, 1996),
308 assuring that stock organisms are reliable.
309
310 *3.3. Subcellular level endpoints*
311 Several endpoints have been evaluated in multigenerational tests with *Daphnia* sp.
312 such as mortality, reproduction or body length (Jacobasch et al., 2014; Kim et al., 2012;
313 Sánchez et al., 2004), however the study of physiological pathways, such as
314 neurotransmission capabilities, detoxification potential or antioxidant capacity is less
315 common. Carbamate pesticides are known to inhibit the ChE activity in *D. magna*
316 (Barata et al., 2004). Though, in the present study ChE levels were significantly higher
317 for the population maintained in CBZ (Dph_CBZ) than for those in clean medium
318 (Dph_Clean) (two-way ANOVA, $F_{1,39} = 11.737$, $p<0.05$) (Table 4 SD), at all
319 generations (Fig. 4a). An increase in the ChE activity in *D. magna* exposed to low
320 concentrations of cadmium and the carbamate propoxur has been already reported
321 (Jemec et al., 2007a; Printes and Callaghan, 2004). Increases in ChE levels at low doses
322 might be explained by compensatory mechanisms after the disruption of homeostasis
323 (Calabrese and Baldwin, 2003). Andrade et al. (2016) observed an increase in ChE
324 activity after exposure of zebrafish (*Danio rerio*) embryos to CBZ, which the authors
325 hypothesized to be related with apoptosis mechanisms, and has also been associated to
326 the induction of gene expression related with apoptosis (Jiang et al., 2014). Although
327 the exact mechanisms are not yet understood, indirect evidences that ChE participates in
328 the regulation of apoptosis and cell proliferation have been theorised (Jiang and Zhang,
329 2008). A similar mechanism related with mediation of cell apoptosis could be playing a
330 role in the ChE activity increase observed in the present study, however additional
331 studies should be performed to confirm this hypothesis. Both exposure and generation
332 showed some significant interaction, indicating that populations responded differently
333 throughout the generations (two-way ANOVA, $F_{3,39} = 3.393$, $p<0.05$) (Table 4 SD).
334 However, throughout the successive generations (F0 to F12), there was an overall trend
335 for the attenuation of this effect with ChE activity in F12 becoming similar between
336 both populations (Fig. 4a). In the present study, variation in ChE activity was observed
337 throughout generations, including in Dph_Clean. Variation in ChE activity amongst
338 individuals of the same species has been observed for *D. magna* in previous studies,
339 including under control conditions. In the literature, under control conditions *D. magna*
340 ChE levels vary from 0.034 to approximately 1.5 nmol/mg prot/min (Jemec et al.,

2007a; Qi et al., 2013), which is in accordance with the ChE activity reported in the present study for daphnids kept in clean medium (Dph_Clean). On the other hand, the values reported here for ChE in Dph_CBZ ranged from 0.74 (F0) to 0.60 nmol/mg prot/min (F12).

Although the main toxicity mechanism of carbamates is usually through ChE inhibition, exposure to carbamate pesticides has shown to trigger also other toxicity effects such as oxidative stress, by inducing generation of reactive oxygen species (ROS) (Milatovic et al., 2006). CAT is an antioxidant enzyme, which is responsible for breaking down hydrogen peroxide into water and molecular oxygen (Claiborne, 1985). CAT activity values reported in the literature for *D. magna* control groups range from 62.4 µmol/mg prot/min (for daphnids with 22 d) to 250 µmol/mg prot/min (for daphnids with 6 d) (Barata et al., 2005; Jemec et al., 2007b). In the present work, CAT levels determined for Dph_Clean (between 25.92 and 39.21 µmol/mg prot/min) were slightly lower comparing with the values reported in literature and were maintained at similar levels from F0 to F12 generations (Fig. 4b). However, several factors might cause this variability, including for instance the type of food provided (*e.g.* algae species), daphnids age and experimental conditions (*e.g.* temperature or photoperiod) (Rose et al., 2004). Comparing both populations, the exposure did not induce statistically differences for CAT levels (two-way ANOVA, $F_{1,37}$ = 0.348, $p>0.05$), though both factors interacted, meaning that populations responded differently throughout the generations due to exposure (two-way ANOVA, $F_{3,37}$ = 10.271, $p<0.001$) (Table 4 SD). Two patterns were observed when comparing both populations within the same generation: in F0 and F6, CAT activity increased upon exposure to CBZ, while in F3 and F12 a decrease was depicted (Fig. 4b). The stimulation in the initial response of F0 daphnids is possibly a typical response to the low CBZ concentration, to reduce oxidative stress (Vega and Pizarro, 2000). This was followed by a decrease in activity, followed by another increase and decrease, possibly meaning that the physiology of the organisms was working towards releasing ROS, whenever needed. CAT activity decreased in the herb fenugreek *Trigonella foenum-graecum* exposed to CBZ (Sangeetha, 2010), which was justified by a decrease in ROS, which was due to the previous activity of other antioxidative stress enzymes. Reduction in CAT activity was also observed in fish tissues (Palanikumar et al., 2014), rats (Adedara et al., 2013) and goats (Prashantkumar et al., 2013) after exposure to CBZ, being surely a dose-related response. Besides this, CAT activity in F3 daphnids exposed to CBZ was highly decreased which can be a

result of a simultaneous activation of another antioxidant defense mechanism, considering that in F6, this enzymatic activity showed a recovery (Sies, 1993; Wu et al., 2011). This may be the case of reduced glutathione (GSH), which is also involved in the removal of hydroperoxides (*e.g.* H_2O_2) (Sies, 1993; Wu et al., 2011), and occurs by its oxidation (mediated by H_2O_2) into glutathione dissulfide (GSSG), therefore reducing the amount of substrate available to induce CAT (Wu et al., 2011).

Regarding GST activity, the reported values in the literature for control groups of *D. magna* varied from 42 nmol/mg prot/min (*D. magna* with 7 d), to 70 nmol/mg prot/min (*D. magna* with 21 d) and reached 235.2 nmol/mg prot/min (age not reported) (Borgeraas and Hessen, 2002; Chen et al., 2005; Domingues et al., 2015). Levels for detoxification looking at GST activity were different between both populations (Dph_Clean and Dph_CBZ) (two-way ANOVA, $F_{1,33} = 4.557$, $p<0.05$) (Fig. 4c and Table 4 SD). Both factors, generation and exposure, interacted, indicating that populations responded differently throughout generations (two-way ANOVA, $F_{3,33} = 2.951$, $p<0.05$) (Table 4 SD). This enzyme plays an important role in cellular detoxification processes of several chemicals and defense against peroxidative products of DNA (Henson et al., 2001). Pesticides can promote the consumption of glutathione in exposed organisms through a GST-catalyzed reaction in detoxification processes, and therefore GST induction aims to protect the organism (Ezemonye and Tongo, 2010; Timur et al., 2002). A multigenerational experiment with *D. magna* exposed to microcystins showed that upon parental exposure for 7 d, a higher GST activity in their offspring was observed when compared to those from controls (Ortiz-Rodriguez et al., 2012).

In the case of LPO, both populations (Dph_Clean and Dph_CBZ) showed differences (two-way ANOVA, $F_{1,39} = 12.957$, $p<0.001$) and there was an interaction between generations and exposure, meaning that, throughout the generations, both populations reacted differently for this biomarker (two-way ANOVA, $F_{3,39} = 6.612$, $p<0.001$) (Fig. 4d and Table 4 SD). In the F0 generation, a decrease in LPO in offspring from Dph_CBZ was observed, comparing with Dph_Clean. Vernouillet et al. (2010) observed a similar decrease in lipid peroxidation when exposed the crustacean *Thamnocephalus platyurus* to the pharmaceutical carbamazepine and suggested that carbamazepine might have preventing fatty acid oxidation in the membranes, by acting as a radical scavenger or by directly downregulate the cytosolic phospolipase A_2 activity. However, in the F6 generation differences in LPO between Dph_Clean and

Dph_CBZ were atenuated (Fig. 4d). In the last generation tested (F12), there was a slightly increase in LPO for nenonates of Dph_CBZ comparing with Dph_Clean. This seems to indicate an imbalance in organisms redox equilibrium towards a situation of oxidative stress as previously described in several organisms (including the european eel and collembola) when exposed to harbor water, carbamazepine, fluoxetine and nanoparticle fullerene C60 (Ahmad et al., 2004; Oliveira et al., 2015; Zhu et al., 2006).

In the study of Palanikumar et al. (2014), the milkfish *Chanos chanos* was exposed to CBZ and chlorpyrifos and a relationship between DNA damage and the fluctuation in antioxidant enzymes responses might exist. In addition, an increase in DNA damage in *D. magna* was already reported from generation F0 to F12 under a similar approach, where daphnids were exposed to CBZ in multigenerational experiment as well (Silva et al., 2017), yet such straight relationship between DNA damage with antioxidant enzymes could not be established.

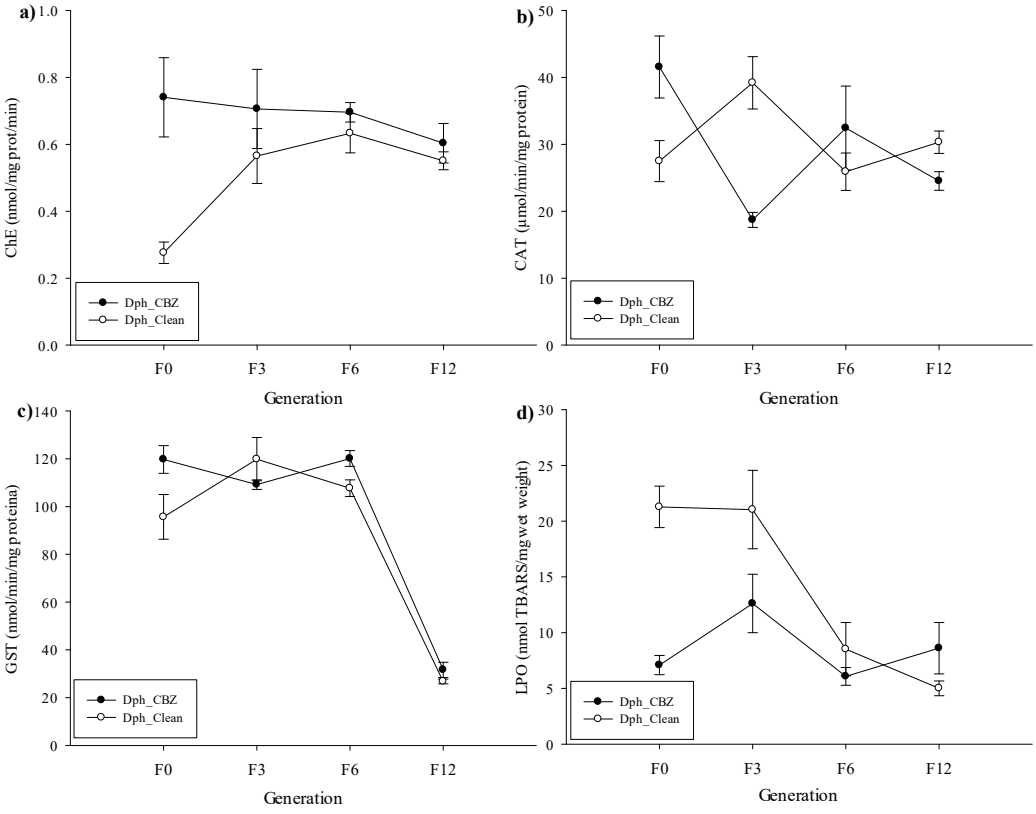

Figure 4. Biomarkers activities in *Daphnia magna* exposed (Dph_CBZ, black dots) and non-exposed (Dph_Clean, white dots) to carbendazim throughout generations: **a)** Cholinesterase (ChE) activity **b)** Catalase (CAT) activity **c)** Glutathione *S*-transferase (GST) activity and **d)** Lipid peroxidation (LPO) rate (n=5). Data are expressed as mean values and standard error.

When under stress and in order to survive, organisms undergo numerous alterations at a low level of biological organization. These alterations include metabolic changes that may end up affecting their energy-reserve fraction and energy consumption (Jeon et al., 2013; Vandenbrouck et al., 2009). Considering the multigenerational effects observed in several life traits, some energy-related parameters were measured in different generations as an attempt to detect and track possible CBZ induced changes in resource allocation. Carbohydrates, which are considered the first energy fraction to be consumed, presented an almost similar pattern between both isoclonal populations Dph_Clean and Dph_CBZ (two-way ANOVA, $F_{1,23} = 0.167$, $p>0.05$) (Fig. 5a and Table 5 SD), with no interaction between both factors, generations and exposure (two-way ANOVA, $F_{3,23} = 2.202$, $p>0.05$) (Table 5 SD). Carbohydrates contents in Dph_Clean neonates are within the range of those found in literature, which vary highly between 199 to 2054 mJ/organism (for neonates <24h exposed for 48h/96h) (De Coen and Janssen, 1997). This variability may be justified by the different food sources and quantities provided to organisms, along with changes in temperature and dissolved oxygen, among others (Bergman Filho et al., 2011) .

For the lipids reserves, a similar pattern was observed for both populations Dph_Clean and Dph_CBZ (two-way ANOVA, $F_{1,22} = 0.113$, $p>0.05$) and no interaction between both factors (generations and exposure) was found (two-way ANOVA, $F_{3,22} = 1.334$, $p>0.05$) (Fig. 5b and Table 5 SD). Lipidic levels obtained in this work for daphnids in clean medium (Dph_Clean) are within the same range reported in literature for the *Daphnia* species (approx. 1000 mJ/organism) (Bergman Filho et al., 2011).

A similar trend was obtained for proteins between Dph_Clean and Dph_CBZ populations (two-way ANOVA, $F_{1,23} = 0.00115$, $p>0.05$) (Fig. 5c and Table 5 SD) with no interaction between both factors, generations and exposure (two-way ANOVA, $F_{3,23} = 3.129$, $p>0.05$) (Table 5 SD). In literature, protein values for *D. magna* in control situation range from to 1694 mJ/organism to 5518 mJ/organism (exposed for 48h) (De Coen and Janssen, 1997). The Dph_Clean population presented protein values ranging from 2000 mJ/org to 3000 mJ/org, which were maintained throughout generations, as well as those for the population exposed to CBZ. This was somehow contrary to the expected and reported by several authors for several species, where an increase in protein content was observed for *D. magna*, *Danio rerio* and *E. albidus* exposed to lindane, effluents and also CBZ, respectively (De Coen and Janssen, 2003a; Novais and Amorim, 2013; Smolders et al., 2003). In addition, Kim et al. (2014) studied the effects

461 of tetracycline in four generations of *D. magna*; depletions in proteins, carbohydrates
462 and lipid reserves were found in consequence of the stress caused by tetracycline.
463 However, throughout the generations, these reductions were recovered (comparing with
464 the control group), suggesting some adaptation (Kim et al., 2014).

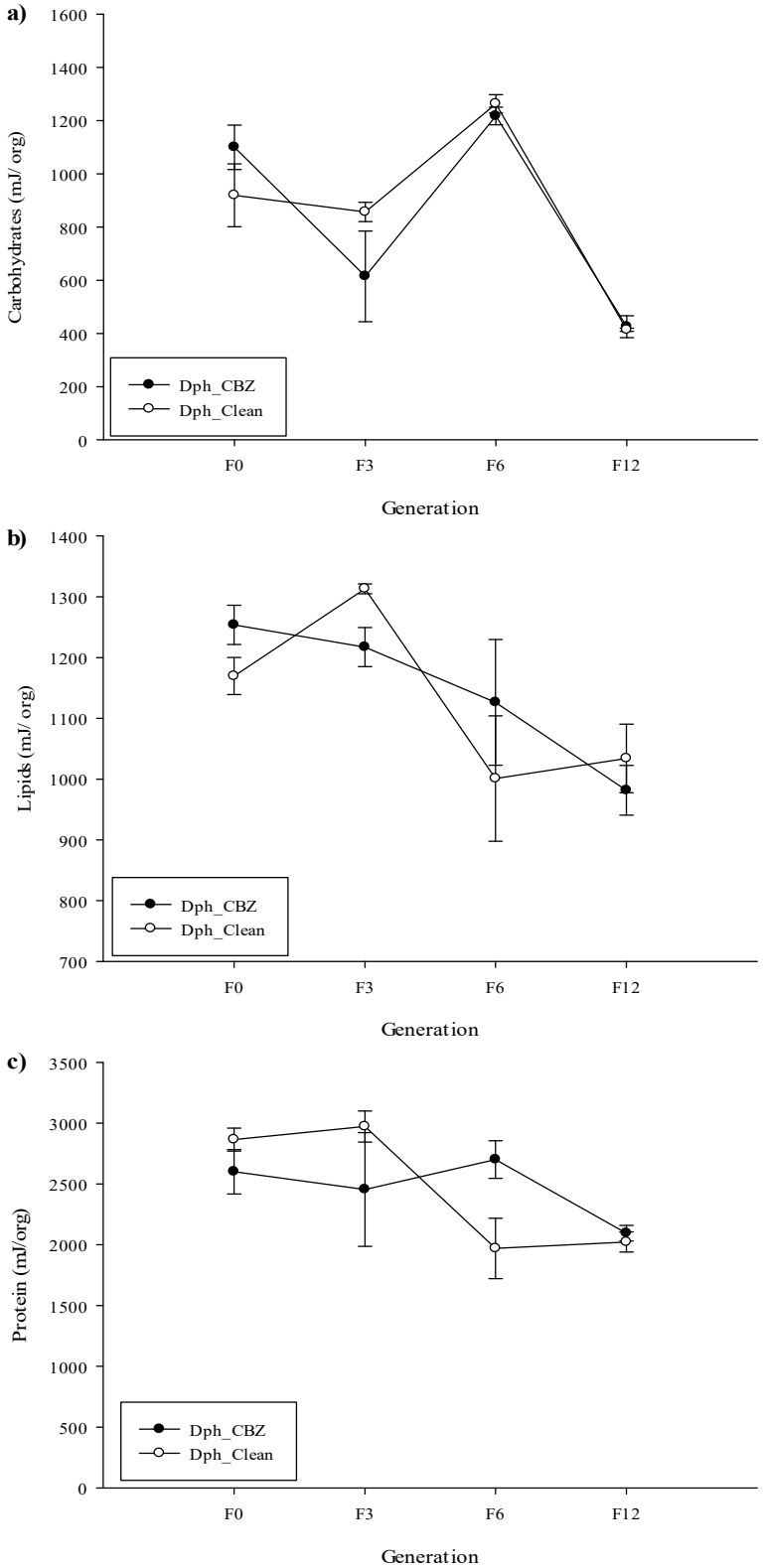

Figure 5. Energy-related parameters in *Daphnia magna* exposed (Dph_CBZ, black dots) and non-exposed (Dph_Clean, white dots) to carbendazim throughout generations: **a)** Carbohydrates **b)** Lipids and **c)** Protein contents (n=3). Data are expressed as mean values and standard error.

This lack of differences in lipidic, protein and carbohydrates contents (Table 5 SD), derive similar Energy available (Ea) or Energy consumed (Ec). While for Ea no interaction between generations and exposure was obtained, the same was not true for Ec (Table 6 SD; Fig. 6). Although no differences in patterns were observed for energy reserves within generations and exposure to CBZ, a trade-off seemed to have occurred, while looking at *r* and organism's longevity. Several examples found in literature reported that *D. magna* showed an ability to switch its life history responses while exposed to stressors (Minguez et al., 2015), and in the present study reproduction was more favoured than survival. Different patterns may be justified by the chemical nature, but also the level of concentration used should be considered. In the present study, the concentration used is a NOEC for reproduction in a 21d exposure. In addition, in the present study, some differences were observed in terms of oxidative stress related biomarkers, showing that in some cases enzymatic activities were activated to achieve homeostasis, while decreasing their activity afterwards, or being compensated by other enzymatic processes within the same molecular pathway. These physiological processes might have helped the organism to maintain a healthy status, and few effects depicted under a population level.

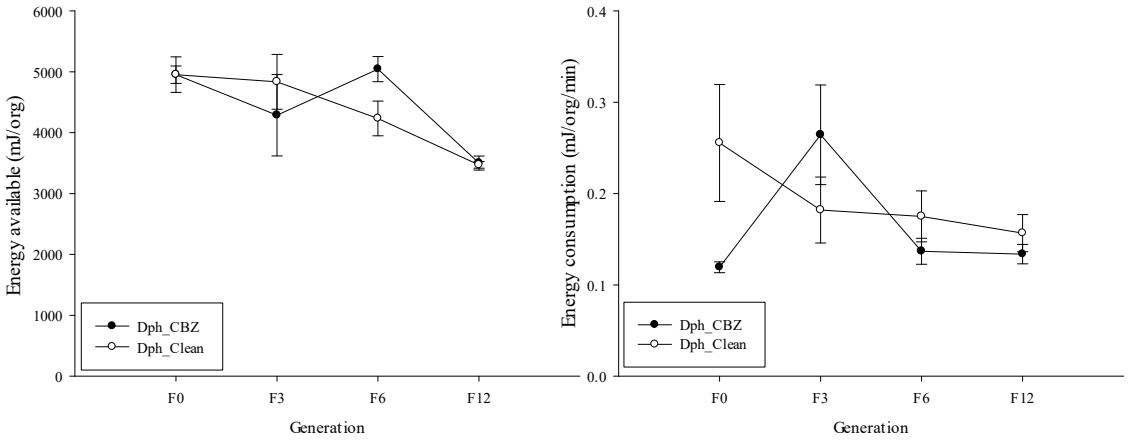

Figure 6. Energy-related parameters on individuals of *Daphnia magna* exposed (Dph_CBZ, black dots) and non-exposed (Dph_Clean, white dots) to carbendazim throughout generations. Data are expressed as mean values and standard error.

The variability observed in several parameters recorded for *D. magna* under control exposure throughout the generations is a pattern that should be further discussed

and not ignored. Besides the enlightenments referred previously (*e.g.* algae quality), Traudt et al. (2016) also demonstrated that exposing daphnids to cadmium using a narrow age window of less than 24h reflected an EC50 value 10 times different between them (when comparing daphnids with 0-4h and daphnids with 20-24h). In the present work, some of the observed effects can be attributed partly to inter-generational environmental and natural variation. In fact, inter-generational effects due to, for instance, changes in parental food environment have already been demonstrated (Plaistow et al., 2005). In the same study, evidences that life history traits are the result of interactions between past but also present environments and that these traits vary from one generation to the subsequent one were demonstrated. In addition, trade-offs between endpoints were found (as observed in the present work) and changed depending on the high (trade-off between fecundity and survival) or low food supply (trade-off between age and size) (Plaistow et al., 2005). Variability in responses has been discussed since the 90's for daphnids, where apart from the genotype and environmental variables and their interaction, an additional variable, the "residual variability component" is present (Soares et al., 1992). This former variable may include for instance measurement/pure errors and others unexplained/natural (Falconer and Mackay, 1996).

4. Conclusions

The multigenerational exposure of *D. magna* to a NOEC equivalent concentration of CBZ induced low effects but provided useful information to understand how populations react to long-term exposure to chemicals. One of the first highlights derived from the present study is that the continuous exposure to CBZ did not induce changes in the intrinsic rate of natural increase (*r*) but deeply affected their longevity, with a notorious decrease in the lifespan found in daphnids exposed after 12 generations to CBZ. Considering that energy related-parameters showed no significant differences between both populations, a trade-off in energy allocation possibly occurred, with more energy being allocated in the reproduction of daphnids, diminishing energy available for survival in a long term. Energy was also partly allocated to detoxification, when looking at biomarkers' patterns. Although ChE, GST and LPO showed differences between clean and exposed isoclonal populations, the identification of a clear detoxification mechanism could not be depict.

Although *D. magna* is a parthenogenic organism generally expected to show low variability in responses, several life-trait parameters within generations in control exposures presented some inconsistency.

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Data availability— Data and calculation tools are available from the corresponding author (ritas@ua.pt).

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